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miRNA Detection

Direct Quantitative Analysis of Multiple miRNAs (DQAMmiR)**

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MicroRNAs (miRNAs) are short RNA molecules (18–25 nucleotides) that were recently proven to play an important role in the regulation of cellular processes,[1,2] and their abnormal expression is associated with pathologies such as cancer.[3,4] A change in the cellular status is typically associated with a simultaneous change in the level of several miRNAs.[5–11] For example, abnormal expression of two miRNAs was found to be indicative of colorectal cancer in humans.[12] Therefore, both the study of the biological role of miRNA and the use of miRNA for informative disease diagnostics require accurate quantitative analysis of multiple miRNAs. Most methods of miRNA detection are indirect (e.g. PCR, microarrays, SPR, next generation sequencing, etc.), that is, they require chemical or enzymatic modifications of miRNA prior to the analysis.[13–16] Not only do these modifications make the analysis more complex and time-consuming but they also reduce the accuracy of the method owing to different efficiencies for modifications of different miRNAs.[17–19] There are a few direct methods that do not require any modification of the target miRNA. Northern blotting does not require any modifications, however, the method can be tedious and although it can be quantitative its sensitivity is limited. Signal-amplifying ribozymes, in situ hybridization, bioluminescence detection, and two-probe single-molecule fluorescence are other direct miRNA detection methods[20–24] however, the first two methods are only semi-quantitative while the latter two can hardly be used for multiple miRNAs. Cheng et al. used rolling-circle amplification (RCA), which does not require modification of the miRNA to detect low concentrations of miRNA and can be run in parallel; however, the process is tedious taking over 8 h and the amplification step can potentially lead to biases in quantitation.[25] Thus, there is currently no method for direct quantitative analysis of multiple miRNAs. Herein we report the first direct quantitative analysis of multiple miRNAs (DQAMmiR). DQAMmiR uses miRNAs directly, without any modification, and accurately determines concentrations of multiple miRNAs without the need for calibration curves. This approach was achieved using a capillary-electrophoresis-based hybridization assay with an ideologically simple combination of two well-known separation-enhancement approaches: 1) drag tags on the DNA probes,[26,27] and 2) single strand DNA binding protein (SSB) in the buffer.[28] In this proof-of-principle work, we developed DQAMmiR for three miRNAs (mir21, 125b, 145) known to be deregulated in breast cancer. DQAMmiR opens the opportunity for simple, fast, and quantitative fingerprinting of up to several tens of miRNAs in basic research and clinical applications. The availability of suitable commercial instruments for DQAMmiR makes the method practical for a large community of researchers.

We based DQAMmiR upon a classical hybridization approach, in which an excess of labeled DNA probes are bound to their complementary miRNA targets. Electrophoresis can be used to efficiently separate oligonucleotides,[29] but simultaneously separating the hybrids from each other and from the unbound probes is challenging and so far has not been achieved.[30] We solved the separation problem through a combination of two well-known mobility-shift approaches: 1) drag tags on the probes[26] and 2) single strand DNA binding (SSB) protein in the buffer.[28] This hypothetical approach is illustrated in Figure 1, in which the miRNAs and their complimentary ssDNA probes are shown as short lines of the same color, drag tags are shown as parachutes, fluorescent labels are shown as small green circles, and SSB is shown as a large black circle. In the hybridization step, an excess of the probes is mixed with the miRNAs, thus leading to all miRNAs' being hybridized but with some probes left unbound to miRNA. A short plug of the hybridization mixture is introduced into a capillary prefilled with an SSB-containing buffer. SSB binds all ssDNA probes but does not bind the double-stranded miRNA–DNA hybrid. When an electric field is applied, all SSB-bound probes move faster than all the hybrids (SSB works as a propellant).[28] Different drag tags make different hybrids move with different velocities. SSB-bound probes, however, can move with similar velocities if the drag tags are small with respect to SSB. In such a case, a fluorescent detector at the end of the capillary generates separate signals for the hybrids and a cumulative signal (one peak or multiple peaks) for the excess of the probes. The amounts of the different miRNAs are finally determined from integrated signals (peak areas in the graph) by a simple mathematical approach. We reserve the term of direct quantitative analysis of multiple miRNAs and its abbreviation of DQAMmiR for the specific approach described above.

To experimentally test the viability of our hypothetical DQAMmiR, we decided to use three miRNAs known to be deregulated in breast cancer: mir21 (5′-UAGCUUAUCAGA CUGAUGUUGA-3′), mir125b (5′-UCCCUGAGACC-CUAACUU GUGA-3′), and mir145 (5′-GUCCA-GUUUUCCCAGGAAUCCC U-3′). Three ssDNA probes were designed and all are labeled with Alexa 488 at the 5' end; the 3' end was reserved for drag tags. To separate the three hybrids we needed only two probes modified with drag

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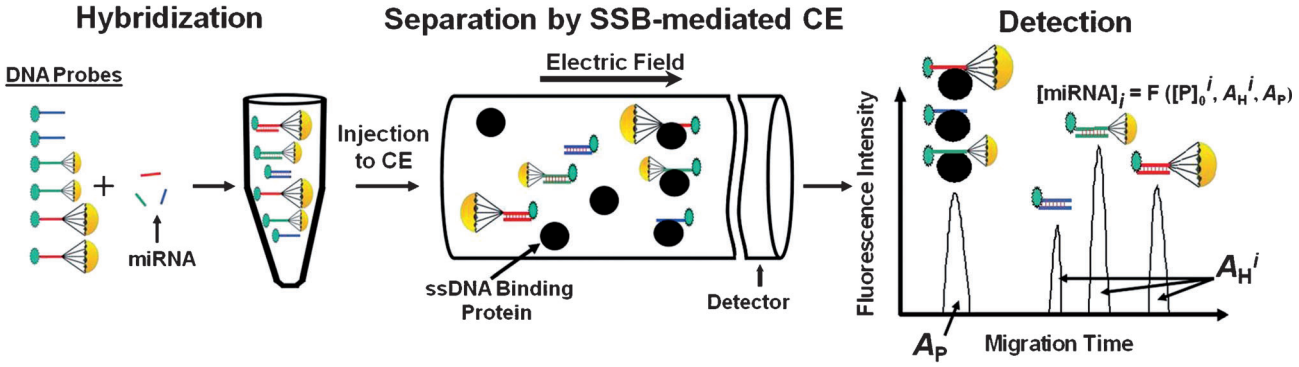

Figure 1. Schematic representation of the direct quantitative analysis of miRNA. See text for details.

tags; one probe could be without a drag tag. In the proof-of-principle work, we chose to use the two simplest available drag tags: a hairpin formed by a DNA extension at the 3′ end of the probe and biotin covalently attached to the 3′ end. The probe for mir21 was the one with the hairpin, which is formed by the following italicized extension: 5′-Alexa 488-TCAACATCAGTCTGATAAGCTA*GCGCGCTTTGCGCGC*-3′. The probe for mir125b was the one with no tag: 5′-Alexa 488-TCACAAGTTAGGGTCTCAGGGA-3′. The probe for mir145 was the one with biotin: 5′-Alexa 488-AGGGATTCCTGGGAAAACTGGAC-Biotin-3′.

The experimental details are described in the Supporting Information. Briefly, the miRNAs were hybridized with the probes in the incubation buffer (50 mM of Tris-Ac, 50 mM of NaCl, 10 mM of EDTA, pH 7.8), containing fluorescein as an internal standard, by first increasing the temperature to a denaturing 80 °C, then lowering it to 37 °C at a rate of 20 °C min^{-1} and finally keeping it at 37 °C for one hour to allow complete hybridization. To minimize miRNA degradation, a nuclease-free environment was used while handling the miRNA samples. The structures of the three hybrids formed are schematically depicted in Figure 2a. The separation was carried out in a bare fused-silica capillary with a positive electrode at the injection end of the capillary. The capillary was prefilled with the SSB-containing buffer (25 mM of sodium tetraborate at pH 9.3 supplemented with 50 nM of SSB). Under such conditions, an electroosmotic flow occurred, and moved negatively charged hybrids and probes to the detection end of the capillary, where the negative electrode was situated. A commercial CE instrument with fluorescence detection suitable for the Alexa488 label on our probes was used. Samples were injected by a pressure pulse of 0.5 psi for 5 seconds, the volume of the injected sample was ≈6 nL. Electrophoresis was driven by an electric field of 500 V cm^{-1} with a capillary coolant temperature set at 20 °C.

Figure 2b shows the result of the electrophoretic separation in DQAMmiR; this result agrees with the hypothesis depicted in Figure 1. SSB bound the excess probes and increased their mobility, thus generating two adjoining peaks at approximately 3.4 and 3.6 minutes. The hybrids had no ssDNA section accessible for SSB to bind and, therefore, SSB did not affect their mobility. The negatively charged hairpin slowed down the mir21 hybrid, while neutral biotin increased the velocity of the mir145 hybrid with respect to the mir125b

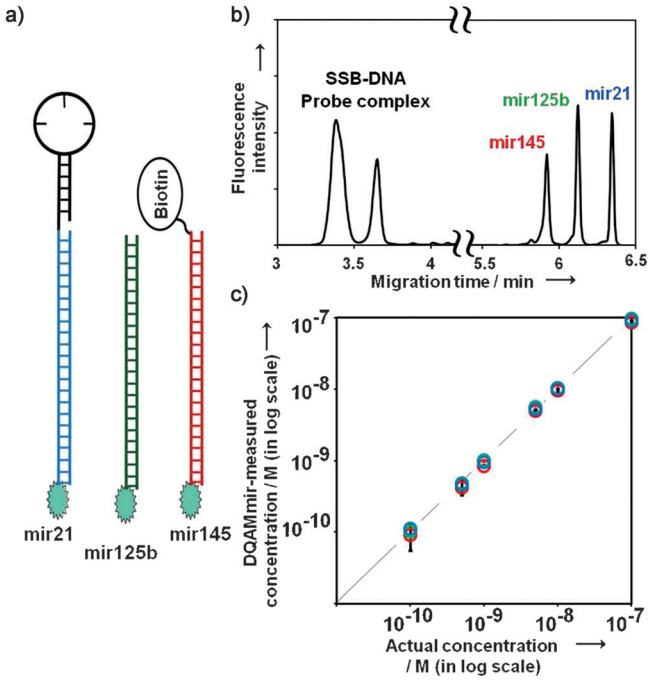

Figure 2. a) Structures of three miRNA–DNA probe hybrids. b) CE separation of the three hybrids from each other and from the excess probe facilitated by the drag tags on mir21 and mir145 and the SSB in the buffer. The concentrations of miRNAs were 5 nM each and the concentration of the probes were 50 nM each. c) Quantitative properties of the analysis utilizing data from b at concentrations of miRNAs varying from 100 pM to 100 nM (three repetitions) and processed with Equation (1). Different concentrations of miRNA were prepared by serial dilution of a stock solution. The concentration of the stock solution was determined by light absorbance at 260 nM. Standard deviation of DQAMmiR-measured miRNA concentrations was 6.6%.

hybrid. All hybrid peaks were perfectly resolved and their areas could be accurately determined, which, in turn, allowed us to determine the quantities of the three miRNAs. The time window between the SSB-bound probes and the hybrids was 2 minutes. With the observed peak widths of the hybrids, the 2 minute window is sufficient to resolve a maximum of approximately 20 peaks. While this maximum can be increased by optimizing the separation conditions it is unlikely to exceed 30–40 peaks. This range is the electrophoresis-associated limit for the maximum number of

miRNAs that can be analyzed by DQAMmiR with fluorescence detection in a single spectral channel.

One of the major requirements of miRNA analysis is selectivity; any miRNA detection method should be able to discriminate miRNA from a similar sequence that differs by a single nucleotide. Such selectivity is typically based on the difference in melting temperatures between the full-match and single-nucleotide-mismatch hybrids. We studied the selectivity of a single DNA probe, and confirmed that increasing the temperature of the electrolyte to above the melting temperature of the single-nucleotide-mismatch hybrid, but to below the melting temperature of the perfect-match hybrid, completely eliminated the peak from the mismatch while not affecting the peak of the match. Moreover, owing to the thermal stability of SSB, our concept worked equally well at the elevated temperature (see the Supporting Information). Thus, DQAMmiR has the potential for single-nucleotide sensitivity, which is required for miRNA detection in biological samples.

To determine the quantities of the individual miRNA molecules from the experimental data, similar to that shown in Figure 2b, we developed a mathematical approach for DQAMmiR; this mathematical approach does not require the resolution of SSB-bound probes and takes into account a potential change in the quantum yield of fluorescence of the probe upon its binding to miRNA or SSB. The derivations can be found in the Supporting Information. Here we present the resulting equation for the calculation of the concentration of the i-th miRNA in the hybridization mixture containing N miRNAs:

$$[\mathrm{miRNA}]^i = \frac{A_\mathrm{H}^i \sum_i^N [\mathrm{P}]_0^i \, q_\mathrm{P}^i}{q_\mathrm{H}^i \left[\left(\sum_i^N A_\mathrm{H}^i \, q_\mathrm{P}^i / q_\mathrm{H}^i \right) + A_\mathrm{P} \right]} \quad (1)$$

$[\mathrm{P}]_0{}^i$ is the total concentration of the i-th probe (composed of the hybrid and the miRNA-unbound probe), A_H is the area corresponding to the i-th hybrid, A_P is the cumulative area of the excess probe, $q_\mathrm{H}{}^i$ is the relative quantum yield of the i-th hybrid with respect to that of the free probe, and $q_\mathrm{P}{}^i$ is the relative quantum yield of the i-th probe in the presence of SSB with respect to that of the free probe. To have a universal equation that can be used when additional DNA probes are introduced to detect an even greater number of miRNA, the peak areas of all the SSB-bound probes were combined to form A_P. Refer to the right-hand part of Figure 1 for a better understanding of the area assignments.

Equation (1) was used to determine the amounts of miRNA in the experiment shown in Figure 2b (the quantum yields were determined in separate experiments described in the Supporting Information). The results of the calculations shown in Figure 2c demonstrate the high accuracy (94%) and great signal linearity ($R = 0.9999$) of the DQAMmiR method in the range of at least three orders of magnitude. It is important to emphasize that DQAMmiR does not require calibration curves.

After proving the concept of DQAMmiR, we tested the method for its tolerance to a complex biological matrix. A sample was made of the three miRNAs added to the *E. coli* cell lysate, supplemented with fluorescein as an internal standard (to ensure controlled injection of the relatively viscous crude cell lysate), and masking RNA and DNA (to prevent degradation of miRNA and DNA probes). The hybridization mixture was prepared, processed, and analyzed in the way described above for pure solutions of miRNA. Figure S2 in the Supporting Information compares the results of DQAMmiR for the cell lysate and for a pure buffer, as the sample matrices. Qualitative comparison of the data shows only insignificant differences. Moreover, calculations of miRNA concentrations (Table S3 in the Supporting Information) also produce similar results, thus confirming that neither the cell lysate nor the masking DNA and RNA significantly affected the results, and that DQAMmiR could be potentially directly used for complex biological samples without RNA extraction or other sample processing.

To test this theory, we used DQAMmiR with a MCF-7 cell lysate sample, which is known to up-regulate mir21 and down-regulate mir125b and mir145. Figure 3 compares the DQAMmiR results for the pure MCF-7 cell lysate and the lysate spiked with the three miRNAs. In the lysate-only sample a peak for the up-regulated mir21 was detected and the concentration of mir21 was determined to be 140 pM. The correctness of this value was confirmed by analyzing 140 pM mir21 in a pure buffer and an identical peak was observed. The peaks of down-regulated mir125b and 145 were below the level of the background noise. This result indicates that available commercial CE instrumentation may not be sensitive enough for DQAMmir of down-regulated miRNAs without their preconcentration. The ultimate solution to this limitation will be the commercialization of instrumentation with single-molecule fluorescence detection, which exists in experimental prototypes.

Below we outline the major features of DQAMmiR and further directions for its development and application. The following major parameters are used to characterize any method of miRNA detection: analysis time, number of miRNAs analyzed simultaneously, specificity, amount of

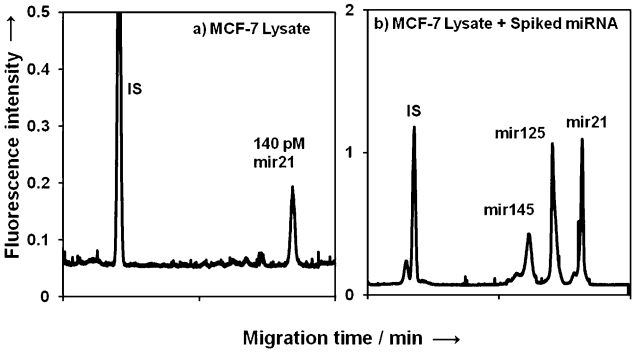

Figure 3. The influence of complex biological matrix on miRNA analysis by DQAMmiR. a) DQAMmiR of three DNA probes at 5 nM each, incubated with MCF-7 cell lysate and masking DNA and RNA in incubation buffer. b) DQAMmiR of three DNA probes at 5 nM each and spiked with 0.5 nM each of mir21, mir125b and mir145 incubated with MCF-7 cell lysate and masking DNA and RNA. IS = internal standard (fluorescein).

sample required, limit of detection, dynamic range, accuracy, and tolerance to biological matrices. With no sample processing involved the analysis time for DQAMmiR is limited by hybridization and separation times only; it is approximately 1.5 hours. The hybridization time can be further shortened by increasing the concentrations of the probes. The resolution between the SSB-bound probes and the hybrids in DQAMmiR ($R_s = 23.4$) roughly suggests that a maximum of approximately 20 miRNAs can be reliably analyzed in a single spectral channel without further optimization of the analysis. This number can be doubled through using two different fluorescent labels and a commercially available instrument with two spectral channels. The design of suitable drag tags and methods for their conjugation to the probes will be crucial for DQAMmiR to reach its electrophoresis limit in terms of the maximum number of miRNAs that can be simultaneously analyzed. With a maximum number of miRNAs analyzed of below 50, DQAMmiR cannot compete with microarrays in extensive miRNA screens, but is suitable for the majority of other applications. This design may take some time and effort but when the tags are developed and validated no further optimization of them will be required. DQAMmiR is capable of detecting differences of a single nucleotide (see Supporting Information), simply by increasing the capillary temperature to above the melting temperatures of all the mismatched hybrids. The temperature should be adjusted to keep the full-match hybrids intact. One analysis consumes a fraction of 1 μL of the sample. The limit of detection of DQAMmiR is restricted by that of CE with fluorescence detection. Commercially available CE instruments have a limit of detection of approximately 10^4–10^5 copies of the target molecule for hybridization assays.[31] Custom-designed detectors can have a limit of detection down to hundred of copies.[32] These limits of detection should be sufficient for the majority of biologically relevant assays.[33] The dynamic range of DQAMmiR is limited by the dynamic range of the linear response of a fluorescent detector, and we found this range to be at least three orders of magnitude (between 10^{-10} and 10^{-7} we have three rather than four orders of magnitude). Our proof-of-principle results demonstrate that the method has an accuracy of approximately 94% and precision of approximately 92%. Our experiment with the cell lysate suggests that DQAMmiR is also highly tolerant to impurities in the sample, thus making the method applicable to crude biological samples.

To conclude, DQAMmiR is the first approach that requires no miRNA modification in the sample, while being quantitative and applicable to multiple miRNAs. With its characteristics, DQAMmiR has a potential of becoming the major tool for quantitative analysis of miRNAs in vitro for all applications but extensive screens.

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[1] B. D. Aguda, Y. Kim, M. Piper-Hunter, A. Friedman, C. B. Marsh, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19678–19683.
[2] M.-R. Suh, Y. Lee, J. Y. Kim, S.-K. Kim, S.-H. Moon, J. Y. Lee, K.-Y. Cha, H. M. Chung, H. S. Yoon, S. Y. Moon, V. N. Kim, K.-S. Kim, *Dev. Biol.* **2004**, *270*, 488–498.
[3] R. Visone, L. Z. Rassenti, A. Veronese, C. Taccioli, S. Costinean, B. D. Aguda, S. Volinia, M. Ferracin, J. Palatini, V. Balatti, H. Alder, M. Negrini, T. J. Kipps, C. M. Croce, *Blood* **2009**, *114*, 3872–3879.
[4] G. A. Calin, C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, C. M. Croce, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15524–15529.
[5] M. V. Iorio et al., *Cancer Res.* **2005**, *65*, 7065–7070.
[6] J. Lu, G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz, T. R. Golub, *Nature* **2005**, *435*, 834–838.
[7] P. T. Nelson, D. A. Baldwin, L. M. Scearce, J. C. Oberholtzer, J. W. Tobias, Z. Mourelatos, *Nat. Methods* **2004**, *1*, 155–161.
[8] W. Li, L. Xie, X. He, J. Li, K. Tu, L. Wei, J. Wu, Y. Guo, X. Ma, P. Zhang, Z. Pan, X. Hu, Y. Zhao, H. Xie, G. Jiang, T. Chen, J. Wang, S. Zheng, J. Cheng, D. Wan, S. Yang, Y. Li, J. Gu, *Int. J. Cancer* **2008**, *123*, 1616–1622.
[9] Y. Murakami, T. Yasuda, K. Saigo, T. Urashima, H. Toyoda, T. Okanoue, K. Shimotohno, *Oncogene* **2006**, *25*, 2537–2545.
[10] A. J. Schetter, S. Y. Leung, J. J. Sohn, K. A. Zanetti, E. D. Bowman, N. Yanaihara, S. T. Yuen, T. L. Chan, D. L. W. Kwong, G. K. H. Au, C.-G. Liu, G. A. Calin, C. M. Croce, C. C. Harris, *JAMA J. Am. Med. Assoc.* **2008**, *299*, 425–436.
[11] N. Yanaihara, N. Caplen, E. Bowman, M. Seike, K. Kumamoto, M. Yi, R. M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G. A. Calin, C.-G. Liu, C. M. Croce, C. C. Harris, *Cancer Cell* **2006**, *9*, 189–198.
[12] M. Z. Michael, S. M. O'Connor, N. G. van Holst Pellekaan, G. P. Young, R. J. James, *Mol. Cancer Res.* **2003**, *1*, 882–891.
[13] K. Lao, N. L. Xu, V. Yeung, C. Chen, K. J. Livak, N. A. Straus, *Biochem. Biophys. Res. Commun.* **2006**, *343*, 85–89.
[14] C.-G. Liu, G. A. Calin, B. Meloon, N. Gamliel, C. Sevignani, M. Ferracin, C. D. Dumitru, M. Shimizu, S. Zupo, M. Dono, H. Alder, F. Bullrich, M. Negrini, C. M. Croce, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9740–9744.
[15] S. Fang, H. J. Lee, A. W. Wark, R. M. Corn, *J. Am. Chem. Soc.* **2006**, *128*, 14044–14046.
[16] R. D. Morin, M. D. O'Connor, M. Griffith, F. Kuchenbauer, A. Delaney, A.-L. Prabhu, Y. Zhao, H. McDonald, T. Zeng, M. Hirst, C. J. Eaves, M. A. Marra, *Genome Res.* **2008**, *18*, 610–621.
[17] E. Ohtsuka, S. Nishikawa, R. Fukomoto, S. Tanaka, A. F. Markham, M. Ikehara, M. Sugiura, *Eur. J. Biochem.* **1977**, *81*, 285–291.
[18] L. W. McLaughlin, E. Romaniuk, P. J. Romaniuk, T. Neilson, *Eur. J. Biochem.* **1982**, *125*, 639–643.
[19] E. A. Weiss, G. M. Gilmartin, J. R. Nevins, *EMBO J.* **1991**, *10*, 215–219.
[20] E. Várallyay, J. Burgyan, Z. Havelda, *Nat. Protoc.* **2008**, *3*, 190–196.
[21] J. S. Hartig, I. Grune, S. H. Najafi-Shoushtari, M. Famulok, *J. Am. Chem. Soc.* **2004**, *126*, 722–723.
[22] W. P. Kloosterman, E. Wienholds, E. de Bruijn, S. Kauppinen, R. H. A. Plasterk, *Nat. Methods* **2006**, *3*, 27–29.
[23] K. A. Cissell, Y. Rahimi, S. Shrestha, E. A. Hunt, S. K. Deo, *Anal. Chem.* **2008**, *80*, 2319–2325.
[24] L. A. Neely, S. Patel, J. Garver, M. Gallo, M. Hackett, S. McLaughlin, M. Nadel, J. Harris, S. Gullans, J. Rooke, *Nat. Methods* **2006**, *3*, 41–46.

[25] Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang, Y. Zhang, *Angew. Chem.* **2009**, *121*, 3318 – 3322; *Angew. Chem. Int. Ed.* **2009**, *48*, 3268 – 3272.
[26] J.-I. Won, R. J. Meagher, A. E. Barron, *Electrophoresis* **2008**, *26*, 2138 – 2148.
[27] H. Zhang, X.-F. Li, X. C. Le, *J. Am. Chem. Soc.* **2008**, *130*, 34 – 35.
[28] M. Berezovski, S. N. Krylov, *J. Am. Chem. Soc.* **2003**, *125*, 13451 – 13454.
[29] A. S. Cohen, D. R. Najarian, A. Paulus, A. Guttman, J. A. Smith, B. L. Karger, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 9660 – 9663.
[30] P.-L. Chang, Y.-S. Chang, J.-H. Chen, S.-J. Chen, H.-C. Chen, *Anal. Chem.* **2008**, *80*, 8554 – 8560.
[31] A. A. Al-Mahrouki, S. N. Krylov, *Anal. Chem.* **2005**, *77*, 8027 – 8030.
[32] Y. F. Cheng, N. J. Dovichi, *Science* **1988**, *242*, 562 – 564.
[33] A. W. Wark, H. J. Lee, R. M. Corn, *Angew. Chem.* **2008**, *120*, 654 – 663; *Angew. Chem. Int. Ed.* **2008**, *47*, 644 – 665.

